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Original Paper

Luteolin Modulates SERCA2a Leading to Attenuation of Myocardial Ischemia/Reperfusion Injury via Sumoylation at Lysine 585 in Mice

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Key Words
Serca2a • Ischemia/reperfusion • SUMOylation • Luteolin

Abstract
Background/Aims: The myocardial sarcoplasmic reticulum calcium ATPase (SERCA2a) is a pivotal pump responsible for calcium cycling in cardiomyocytes. The present study investigated the effect of luteolin (Lut) on restoring SERCA2a protein level and stability reduced by myocardial ischemia/reperfusion (I/R) injury. We verified a hypothesis that Lut protected against myocardial I/R injury by regulating SERCA2a SUMOylation. ***Methods:*** The hemodynamic data, myocardial infarct size of intact hearts, apoptotic analysis, mitochondrial membrane potential (ΔΨm), the level of SERCA2a SUMOylation, and the activity and expression of SERCA2a were examined *in vivo* and *in vitro* to clarify the cardioprotective effects of Lut after SUMO1 was knocked down or over-expressed. The putative SUMO conjugation sites in mouse SERCA2a were investigated as the possible regulatory mechanism of Lut. ***Results:*** Initially, we found that Lut reversed the SUMOylation and stability of SERCA2a as well as the expression of SUMO1, which were reduced by I/R injury *in vitro*. Furthermore, Lut increased the expression and activity of SERCA2a partly through SUMO1, thus improving ΔΨm and reducing apoptotic cells *in vitro* and promoting the recovery of heart function and reducing infarct size *in vivo*. We also demonstrated that SUMO acceptor sites in mouse SERCA2a involving lysine 585, 480 and 571. Among the three acceptor sites, Lut enhanced SERCA2a stability via lysine 585. ***Conclusions*:** Our results suggest that Lut regulates SERCA2a through SUMOylation at lysine 585 to attenuate myocardial I/R injury.

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Introduction

In acute myocardial infarction, reperfusion is an efficient method of salvaging the ischemic myocardium, and includes thrombolysis, percutaneous coronary intervention and coronary artery bypass graft surgery. However, reperfusion itself can induce further irreversible cell injury and death. Ischemia/reperfusion (I/R) injury triggers a series of adverse events, such as reperfusion arrhythmia, myocardial systolic/diastolic dysfunction, no reflow and even sudden death [1]. Therefore, it is vital to investigate the pathogenesis of I/R injury and search for effective new drugs to alleviate or avoid myocardial I/R injury. The cellular and molecular biological mechanisms of myocardial I/R injury are complicated and likely involve oxidative stress, calcium overload, cell inflammation, and mitochondrial permeability transition pore opening which initiates apoptosis and necrosis [2, 3]. Of these, calcium overload is probably the main cause of I/R injury [4, 5].

Intracellular calcium concentration is regulated by a group of ion channels, transporters and pumps in a highly coordinated manner. Cardiac sarcoplasmic reticulum calcium ATPase (SERCA2a) plays an important role in myocardial excitation-contraction coupling. As a calcium pump, SERCA2a is responsible for taking up calcium from the cytosol into the sarcoplasmic reticulum (SR). The activity of SERCA2a is dynamically regulated by phospholamban phosphorylation, resulting in enhanced calcium transport into the SR, thereby preparing the next contraction cycle [6]. The SERCA2a promoter is involved in I/R injury and reduced SERCA2a activity is extraordinarily harmful for heart systolic/diastolic function [7–9]. As SERCA2a has an essential effect on intracellular calcium homeostasis, the potential molecular mechanism of SERCA2a in myocardial I/R injury deserves to be explored.

SUMO, a small ubiquitin-like modifier, belongs to a family of ubiquitin-like proteins. SUMO isoforms may have overlapping targets but exhibit preferred substrate specificities. For instance, RanGAP1 and Nkx2.5 are favored by SUMO1 [10]. SUMOylation is a reversible post-translational modification where SUMO is covalently conjugated to the lysine residues of a target by enzymatic reactions [11]. SUMOylation has emerged as a crucial post-translational regulatory mechanism of target proteins in many cellular processes, including protein stability, transcriptional regulation, and response to stress [10, 12]. In the heart, SUMOylation has a crucial impact on normal cardiac development and function [13], as well as adaptation of the heart to pathological stress [14]. It has been reported that enhanced SERCA2a SUMOylation can improve cardiac function via SUMO1 gene transfer in swine with heart failure [15]. The small molecule, N106 (N-(4-methoxybenzo [d]thiazol-2-yl)-5-(4-methoxyphenyl)-1, 3,4-oxadiazol-2-amine has been shown to enhance SERCA2a SUMOylation, resulting in enhanced contractility of the failing heart [16]. However, very few studies have investigated the role of SERCA2a SUMOylation in myocardial I/R injury.

Luteolin (Lut) is a natural soluble flavone present in many plants. Our previous studies have demonstrated that Lut can protect the myocardium from I/R injury by down-regulating microRNA-208b-3p [17] and the PI3K/Akt pathway [18]. Moreover, decreased expression and activity of SERCA2a can be partly reversed by Lut [19, 20]. These studies provide evidence that Lut has a protective effect on heart.

According to previous reported results, Lut has been implicated as a modulator of SERCA2a against myocardial I/R injury. We questioned whether Lut could modulate SERCA2a through SUMOylation *in vivo* and *in vitro*. Thereby, we first determined the influence of Lut on the stability and SUMOylation of SERCA2a. Secondly, the relationship between Lut, SUMO1 and SERCA2a was evaluated by knockdown and over-expression of SUMO1. Thirdly, we determined the putative SUMO conjugation sites in mouse SERCA2a, and detected the interaction between the cardio-protective effect of Lut and these conjugation sites. Thus, these findings may reveal the potential therapeutic targets of an effective drug for the clinical prevention and treatment of myocardial I/R injury.

Materials and Methods

Animal care and reagents

All the animal experiments were approved by the Animal Ethics Committee of Xuzhou Medical University, Xuzhou, China, and conformed to the NIH guidelines (Guide for the care and use of laboratory animals). Male C57BL/6J mice (Xuzhou Medical University) weighing 20–25 g were used in the study. Lut (Fluka; purity > 98%; Sigma-Aldrich, Seelze, Germany) was dissolved in dimethyl sulfoxide (DMSO) and diluted with culture medium to a final concentration of 0.01%, which had no side-effects on the heart [21].

HL-1 cell culture

HL-1 cells, derived from mouse atrial hyperplastic cardiac muscle cells, were cultured in Claycomb Medium (Sigma-Aldrich, Saint Louis, MO, USA) supplemented with 10% fetal bovine serum, 100 μg/ml penicillin/streptomycin, 2 mM L-glutamine, and 0.1 mM norepinephrine. Cells were plated in tissue culture flasks coated an hour previously with gelatin (0.02% w/v) and fibronectin (0.5% v/v) [22].

Simulated I/R model using HL-1 cells

Cells in the different groups were treated as follows: Control (Con) group, cells were cultured in Claycomb Medium at normoxia (21% O_2) and 37°C; Simulated I/R (SI/R) group, cells were grown in Claycomb Medium at normoxia (21% O_2) and 37°C, and then subjected to 2 h hypoxia (1% O_2) followed by 2 h normoxia (21% O_2) [23, 24]; Lut pretreatment (Lut+SI/R) group, cells were pretreated with Lut (8 μM) for 12 h followed by SI/R.

I/R injury in mouse myocardium in vivo

Mice were anesthetized with pentobarbital sodium (50 mg/kg) intraperitoneally. A left thoracotomy was performed followed by opening of the pericardium to expose the heart. An 8-0 silk suture was passed underneath the left coronary artery (LAD, 2 mm inferior to the left atrial appendage) [25]. Occlusion was confirmed when left ventricular color changed from red to pallor and ST segment elevation was seen on ECG. Each group was subjected to 30 min of ischemia. The knot was then released to start reperfusion for 24 h. Reperfusion was identified by the recovery of left ventricular color and the variation in ECG. The Sham group underwent the same procedure with the exception of LAD ligation.

Drug administration

C57BL/6J mice were randomly divided into the sham operation group (Sham), I/R group, I/R+DMSO group, Lut5μg/kg+I/R group, Lut10μg/kg+I/R group, Lut15μg/kg+I/R group, Lut20μg/kg+I/R group and Lut25μg/kg+I/R group. Before I/R, Lut was administered by tail vein injection once a day for 3 days [26]. The Sham and I/R group received 25 μg/kg of normal saline for three days. The I/R+DMSO group received 25 μg/kg of DMSO for three days.

MTT assay of cardiac tissue

An MTT assay was performed to test cell viability in cardiac tissue. Briefly, following 30 min ischemia and 24 h reperfusion, cardiac tissue was weighed and frozen. The hearts were then cut transversely into 2-mm-thick slices, which were incubated with MTT (3 mM) for 30 min at 37°C and homogenized in DMSO (40 ml/g). The supernatant was collected following centrifugation at 1000 × *g* for 10 min. The optical density at 550 nm was read with a microplate reader (Bio-Rad 550, Hercules, CA, USA).

Measurement of plasma lactate dehydrogenase (LDH) level

Following completion of the I/R model, carotid blood was collected from each group. The blood was centrifuged at 1000 × *g* for 10 min, and the concentration of plasma LDH was determined using an LDH activity assay kit (BioVison, Milpitas, CA, USA).

siRNA and plasmid constructions

The siRNA of SUMO1 (si-SUMO1) was designed and synthesized by GenePharma (Shanghai, China), and the sequence of si-SUMO1 was as follows: sense, 5'-CUGAGGACUUAGGCGAUAATT-3'; antisense, 3'-UUAUCGCCUAAGUCCUCAGTT-5'.

The over-expressed SUMO1 plasmid (EGFP-SUMO1) was purchased from GenePharma (Shanghai), and the vector was the pEX-2 plasmid containing enhanced green fluorescent protein (EGFP). Empty vector was used as the control plasmid.

Plasmids encoding wild type (WT) or SUMOylation site mutants of SERCA2a were purchased from Hanbio (Shanghai, China) with mutations at the lysine residue (K) to arginine (R). Point mutantion derivatives of SERCA2a (K585R, K480R, K571R, K585R/K480R, K585R/K571R, K480R/571R and K585R/K480R/571R) and WT were constructed in pIRES-EGFP plasmids. All transfections were performed using Lipofectamine 2000 according to the manufacturer's instructions (Invitrogen, Carlsbad, CA, USA).

Transient transfection with AV-sh-SUMO1

Replication-deficient adenoviral vectors encoding SUMO1 interfering sequence with green fluorescent protein (AV-sh-SUMO1) or green fluorescent protein alone (AV-sh-NULL) were generated by JiMan (Shanghai, China). The sequence of AV-sh-SUMO1 was as follows: sense, 5'-CUGAGGACUUAGGCGAUAATT-3'; antisense, 3'-UUAUCGCCUAAGUCCUCAGTT-5'. AV-sh-SUMO1 or AV-sh-NULL (1×10^{10} plaque-forming units) in a total volume of 10 μl was injected into the free wall of the left ventricle using a 30-gauge needle. The injection site was distributed from the apex upward and three adjacent sites on the left ventricular free wall [25]. Gene transfer was performed 3 days before I/R injury. The efficiency of transfection was determined by observation of myocardial green fluorescence and the detection of SUMO1 expression.

Assessment of area at risk and infarct size

Myocardial infarct size was determined by Evans Blue/triphenyltetrazolium chloride (TTC) staining. At the end of reperfusion, the animals were re-anesthetized with pentobarbital sodium (50 mg/kg) intraperitoneally and intubated. The chest was opened again, and the LAD was re-ligated at the same position. Evans blue dye (0.2 ml of a 2.0% solution) was injected into the left ventricular cavity followed by a cardioplegic solution (10% potassium chloride) to ensure the heart stopped in diastole. The heart was then excised, frozen for 10 min to harden the tissue, and serially sectioned from the apex to the atrioventricular groove into five slices, approximately 1.5 mm in thickness. The slices were incubated with 1% TTC at 37°C for 15 min in the dark. The samples were then fixed in a 10% formalin solution for 2 h. Each section was weighed, digitally photographed and analyzed using Image J software (National Institutes of Health, MD, USA). Evans blue-stained areas were defined as non-ischemic areas. The area of myocardium not stained with Evans blue was defined as the area at risk (AAR). TTC and Evans blue-unstained areas were defined as infarcted zones.

Measurement of hemodynamics

Cardiac function was determined 24 h after reperfusion by a catheter connected to a pressure transducer. In brief, after the mouse was re-anesthetized with 1% isoflurane (v/v), the right carotid artery was isolated and the conductance catheter was inserted which was pre-soaked in NaCl (0.9%, 37°C) for 30 min. After stabilization, the left ventricular systolic pressure (LVSP), left ventricular end-diastolic pressure (LVEDP), maximal rate of pressure development (+dP/dtmax) and maximal rate of pressure decrease (−dP/dtmax) were determined using the BL420s Biological function test system (TaiMeng, Chengdu, China). At the end of experiment, the mice were euthanasia with an overdose of pentobarbital sodium (150 mg/kg).

Detection of apoptotic cells

Cell apoptosis was examined by the Annexin V-APC/propidium iodide (PI) dual staining kit (eBioscience, San Diego, CA, USA). The cells were resuspended in 100 μl binding buffer and incubated with 5 μl Annexin V-APC for 15 min at room temperature in the dark according to the manufacturer's instructions. Following the addition of 5 μl PI, apoptotic cells were evaluated using flow cytometry (Becton Dickinson, San Jose, CA, USA). Annexin V^-/PI^- staining indicated surviving cells. Early apoptosis appeared as Annexin V^+/PI^-. PI^+ cells represented apoptotic cells at the terminal stage or necrotic cells.

Measurement of mitochondrial membrane potential (ΔΨm)

Alteration of ΔΨm was measured by the JC-1 Mitochondrial Membrane Potential assay kit (KaiJi Bio, Nanjing, China). Briefly, JC-1 staining solution (5 μg/ml) was added to six-well plates. The cells were subsequently incubated for 20 min at 37°C, washed twice with JC-1 staining buffer, and then collected to

assess ΔΨm by flow cytometry (Becton Dickinson). JC-1 is present as red fluorescence J-aggregates in cells with a high ΔΨm. A green fluorescent monomer is formed in depolarized mitochondria. The changes in ΔΨm were evaluated by the monomer/aggregate emission ratio.

Quantitative real-time PCR (qRT-PCR)

Total RNA was isolated using TRIzol Reagent (Invitrogen). 2 μg RNA was reverse transcribed with Transcript First-strand cDNA synthesis SuperMix (TransGen Biotech, Beijing, China). The mRNA level was determined by qRT-PCR using an ABI PRISM7900 system (Applied Biosystems, CA, USA). The results were normalized with β-actin. The primer sequences were designed by GenePharma (Shanghai, China) as follows: SERCA2a forward, 5'-CGGTGCCTTTGTTGTCTCCA-3', SERCA2a reverse, 5'-ACCTGACTTTCGTCGGCTGTGT-3'; β-actin forward, 5'-TGAGAGGGAAATCGTGCGTGAC-3', β-actin reverse, 5'-GCTCGTTGCCAATAGTGATGACC-3'.

Western blot

Cells and tissues were lysed with 100 μl ice-cold RIPA lysis buffer (Beyotime, Shanghai, China) supplemented with 1 μl 100 mM phenylmethanesulfonyl fluoride (Beyotime) for 30 min. The total protein extract was collected following centrifugation at 12, 000× *g* for 15 min at 4°C. Protein samples were subjected to SDS-polyacrylamide gel electrophoresis followed by transfer onto polyvinylidene fluoride membranes. Non-specific binding was blocked with Tris-buffered saline/Tween-20 for 3 h at room temperature. The blots were then probed with primary antibodies targeting rabbit anti-SUMO1 (1:2000; Santa Cruz Biotechnology, Santa Cruz, CA, USA), mouse anti-SERCA2a (1:5000; Abcam, Cambridge, UK), mouse anti-Bcl-2, mouse anti-Bax (1:1000; Santa Cruz Biotechnology, Santa Cruz, CA, USA), rabbit anti-caspase-3, rabbit anti-cleaved-caspase-3 (1:1000; Cell Signaling technology, CST, USA) at 4°C overnight. The blots were probed again with anti-mouse-HRP and anti-rabbit-HRP secondary antibodies at 37°C for 1 h. The immunoreactive bands were visualized using an enhanced Chemiluminescence (ECL, Merck Millipore, Billerica, MA, USA) method. Quantification of the blots was measured with Image J software (National Institutes of Health). Monomeric SUMO1 and SUMO1 conjugates, such as SUMO1-RanGAP showed the same trend [27]. SUMO1-RanGAP was used to represent total SUMO1.

Measurement of SERCA2a activity

SERCA2a activity in heart tissue was determined by an ATPase Assay Kit (Jiancheng, Nanjing, China) according to the manufacturer's instructions. ATP can be resolved by ATPase into ADP and Pi, therefore, in our test the quantity of Pi was detected to reflect the ATPase activity. One unit of ATPase activity was defined as the amount of Pi which was resolved by ATPase per milligram tissue protein per hour, and was expressed as μmolPi/mgprot/hour. Protein concentration was measured using the BCA Assay Kit (Beyotime).

Assessment of SERCA2a stability

Cells were treated with culture media containing 100μg/ml cycloheximide (Solarbio, Beijing, China) after Lut treatment and transfection with plasmids for 48 h to inhibit protein synthesis. Protein of cells from each group was extracted on days 0, 3 and 5 afer cycloheximide treatment. Western blot analysis was performed to detect the remaining SERCA2a at different time points.

SUMOylation assay

The cell and tissue lysates were immunoprecipitated using the Catch and Release® Reversible Immunoprecipitation System (Merck Millipore) following the manufacturer's instructions. Extracted proteins (500 μg) were incubated with 2 μg of SUMO1 primary antibody or normal rabbit IgG on a rotator at 4°C overnight. The proteins were eluted by the addition of 70 μl of elution buffer and analyzed by western blot.

Statistical analysis

Data are shown as means ± standard errors of the mean. Statistical analysis was performed by one-way and two-way ANOVA using GraphPad Prism 5.0 software (GraphPad Software, Inc., CA, USA). $P<0.05$ was considered statistically significant.

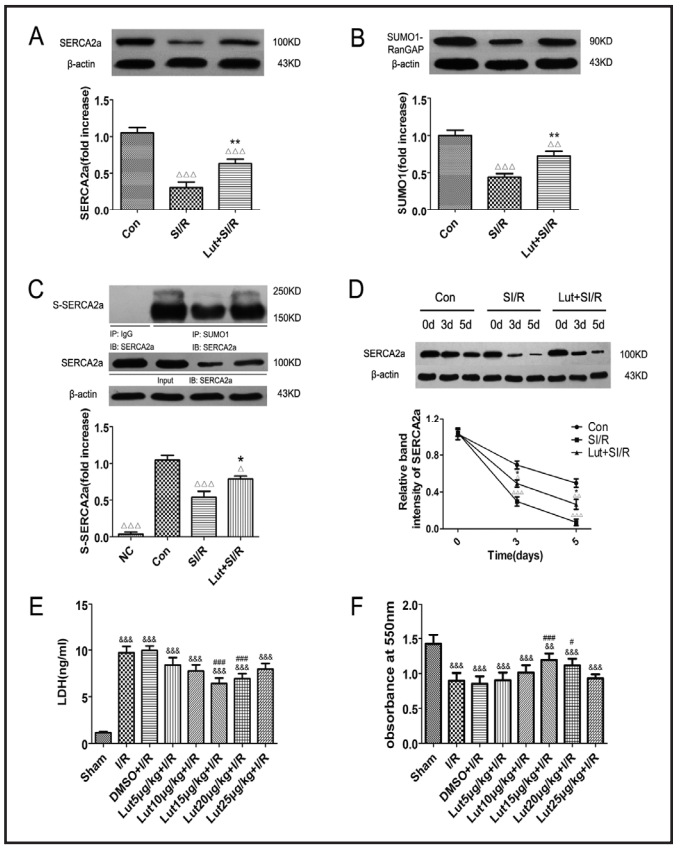

Fig. 1. Lut upregulates SERCA2a and SUMO1 and improves SERCA2a's SUMOylation and stability. (A) SERCA2a protein expression in SI/R process and pretreatment of Lut. (B) SUMO1 protein level in subjected to SI/R injury and pretreated with Lut. (C) The effects of Lut on SERCA2a SUMOylation during SI/R. (D) SERCA2a protein stability in each treatment group. (E) Results of plasma LDH level. (F) Myocardial activity by MTT assay detected at 550nm. Data are presented as mean ± SEM (n=3). ΔP<0.05, ΔΔP<0.01, ΔΔΔP<0.001 versus Con; *P<0.05, **P<0.01 versus SI/R; &&P<0.01, &&&P<0.001 versus Sham; #P<0.05, ###P<0.001 versus I/R.

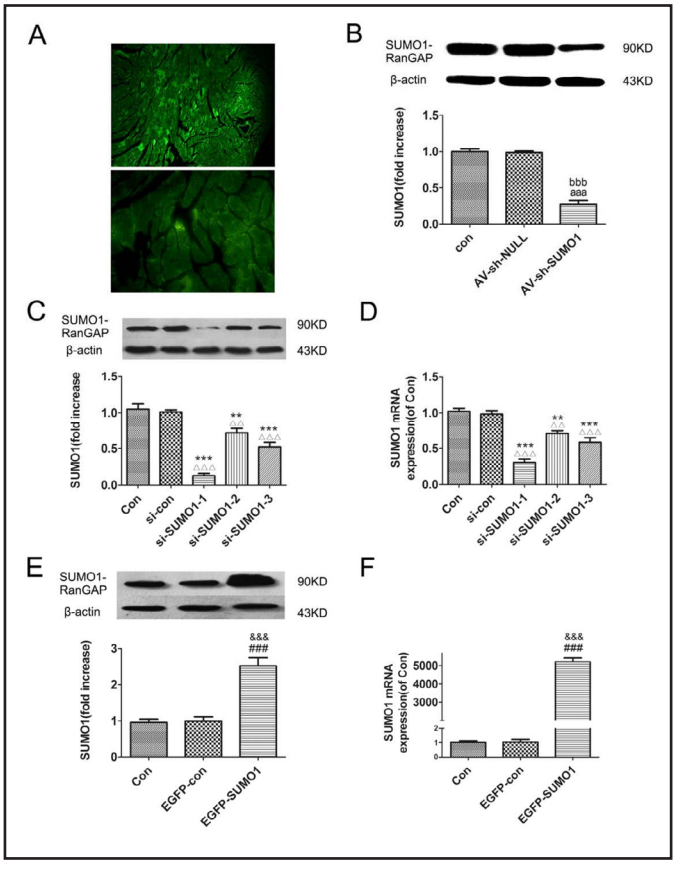

Fig. 2. The filtration and efficiency of AV-sh-SUMO1, si-SUMO1 and EGFP-SUMO1. (A) Myocardial green fluorescence was significantly decreased in AV-sh-SUMO1-injected hearts compared with AV-sh-NULL-injected animals. (B) SUMO1 protein expression in AV-sh-NULL and AV-sh-SUMO1 injected hearts. (C) The effect of transfection with si-SUMO1 on SUMO1 protein level in HL-1 cells. (D) SUMO1 mRNA expression significantly decreased in HL-1 cells transfected with si-SUMO1. (E) The protein level of SUMO1 was increased after EGFP-SUMO1 transfecton in cells. (F) Results of SUMO1 mRNA expression in HL-1 cells transfected with EGFP-SUMO1. Data are presented as mean ± SEM (n=3). aaa P<0.001 versus con; bbb P<0.001 versus AV-sh-NULL; ΔΔP<0.01, ΔΔΔP<0.001 versus Con; **P<0.01, ***P<0.001 versus si-con; ###P<0.001 versus Con; &&&P<0.001 versus EGFP-SUMO1.

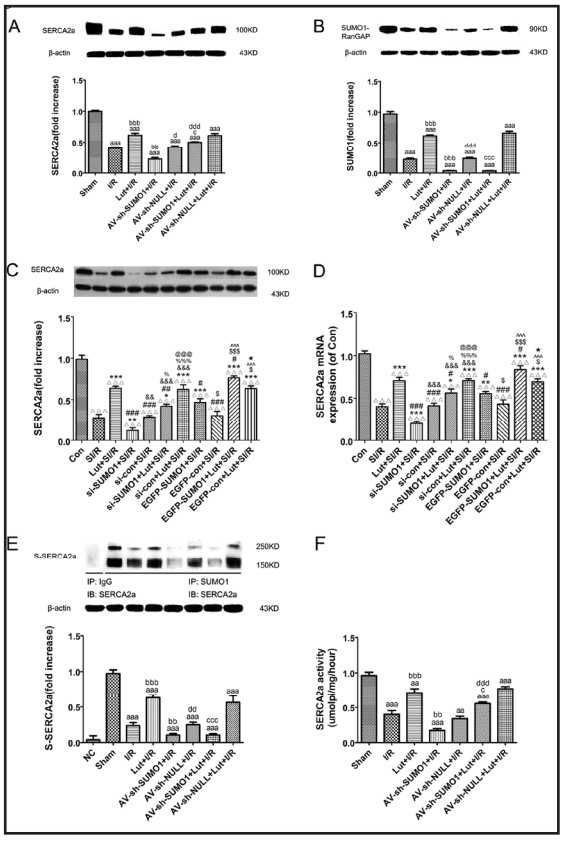

Fig. 3. Expressions of SERCA2a and SUMO1, the level of SERCA2a SUMOylation and SERCA2a activity in each treatment group. (A) The protein level of SERCA2a in vivo with the transfection of AV-sh-SUMO1. (B) SUMO1 protein expression in vivo. (C) HL-1 cells were pre-treated with siRNA to knockdown SUMO1 and plasmids to over-express SUMO1. Cells were then stimulated with SI/R following the pretreatment of Lut. SERCA2a protein level was analyzed by Western blot. (D) Analysis of SERCA2a mRNA expression in each group HL-1 cells by qRT-PCR. (E) The expression of SUMOylated SERCA2a in vivo with the transfection of AV-sh-SUMO1 and Lut pretreatment. (F) The results of SERCA2a activity in vivo. Data are presented as mean ± SEM (n=3). aa P<0.01,aaa P<0.001 versus Sham; bb P<0.01, bbb P<0.001 versus I/R; c P<0.05, ccc P <0.001 versus Lut+I/R; d P<0.05, ddd P<0.001 versus AV-sh-SUMO1+I/R; ΔΔΔP<0.001 versus Con; *P<0.05, **P<0.01, ***P<0.001 versus SI/R; #P<0.05, ##P<0.01, ###P<0.001 versus Lut+SI/R; &&P<0.01, &&&P<0.001 versus si-SUMO1+SI/R; %P<0.05, %%%P<0.001 versus si-con+SI/R; @@@P<0.001 versus si-SUMO1+Lut+SI/R; $P<0.05, $$$P<0.001 versus EGFP-SUMO1+SI/R; ^^^P<0.001 versus EGFP-con+SI/R; ★P<0.05 versus EGFP-SUMO1+Lut+SI/R.

Results

Lut reversed the reduction of SERCA2a, SUMO1 protein expression and increased the SUMOylation and stability of SERCA2a after sI/R injury

SERCA2a and SUMO1 protein expression was detected by western blot and theSUMOylation and stability of SERCA2a were determined in vitro. During SI/R, the protein levels of SERCA2a and SUMO1 decreased compared to the Con group (both P<0.001). Pretreatment with Lut partly reversed the reduction in SERCA2a and SUMO1 protein expression in the SI/R group (both P<0.001) (Fig. 1A–B).

A SUMOylation assay was used to determine the level of SUMOylated SERCA2a (S-SERCA2a). A significant reduction in the level of S-SERCA2a was observed following SI/R (P<0.001). However, following pretreatment with Lut, the expression of S-SERCA2a was partly restored (P<0.05) (Fig. 1C).

To assess whether Lut influenced SERCA2a stability, we first compared the half-life of SERCA2a by performing cycloheximide time-course experiments upon transfection into HL-1 cells. SERCA2a in the SI/R group was less stable (t1/2=2.1 d) than in the Con group where the half-life was estimated to be approximately 4.9 d. Lut pretreatment increased SERCA2a stability which was reduced by SI/R injury (Fig. 1D).

The optimal concentration of Lut in vivo

Compared with the Sham group, the LDH level was significantly increased and MTT level was decreased following I/R injury. Both LDH and MTT level were not significantly different in the I/R group and I/R+DMSO group. Pretreatment with Lut 15 μg/kg and 20 μg/kg decreased LDH level and increased MTT level significantly when compared to the I/R group. Therefore, Lut 15 μg/kg was used in the follow-up experiment (Fig. 1E–F).

Lut increased SERCA2a protein expression partly through SUMO1

The filtration and efficiency of AV-sh-SUMO1, si-SUMO1 and EGFP-SUMO1 were shown in Fig. 2. Myocardial green fluorescence and SUMO1 protein level were significantly decreased in AV-sh-SUMO1-injected hearts compared with AV-sh-NULL-injected animals (Fig. 2A-B). Transfection with si-SUMO1 resulted in significantly decrease in the expressions of SUMO1 protein and mRNA (Fig. 2C-D). The protein and mRNA levels of SUMO1 were increased after EGFP-SUMO1 transfecton in cells (Fig. 2E-F). As shown in Fig. 3, SUMO1 was knocked down and over-expressed to determine the relationship between SERCA2a and SUMO1 in vivo and in vitro. In vitro, SERCA2a and SUMO1 protein level declined following I/R injury ($P<0.001$), and this decrease was partly reversed by pretreatment with Lut ($P<0.001$) (Fig. 3A–B). Compared with the si-Con+SI/R group, the protein expression of SERCA2a was decreased in the si-SUMO1+SI/R group ($P<0.01$) (Fig. 3C). SERCA2a protein level in the si-SUMO1+Lut+SI/R group was increased compared to the si-SUMO1+SI/R group ($P<0.001$), but was decreased when compared to the Lut+SI/R group ($P<0.05$) (Fig. 3C). The level of SERCA2a protein following treatment with AV-sh-SUMO1 in vivo was consistent with the above results (Fig. 3A). The over-expression of SUMO1 up-regulated SERCA2a protein level during I/R injury ($P<0.001$), which was more effective with simultaneous Lut pretreatment ($P<0.001$) (Fig. 3C). The level of SERCA2a mRNA and SERCA2a protein showed the same trend (Fig. 3D).

Lut enhanced SUMOylation of SERCA2a and SERCA2a activity in vivo

I/R injury decreased the expression of S-SERCA2a. Pretreatment with Lut significantly increased S-SERCA2a expression. Compared with the Lut+I/R group, S-SERCA2a level decreased in the AV-sh-SUMO1+Lut+I/R group (Fig. 3E). SERCA2a activity showed the same trend with S-SERCA2a expression (Fig. 3F).

Lut decreased myocardial infarct size and plasma LDH level partly through SUMO1

No difference was found in the AAR among the groups (Fig. 4A–B). A significant increase in both infarct size and LDH level was observed following I/R injury compared to the Sham group. Pretreatment with Lut reduced infarct size and LDH level due to I/R injury. Infarct size and LDH levels in the AV-sh-SUMO1+Lut+I/R group were higher than those in the Lut+I/R group, but lower than those in the AV-sh-SUMO1+I/R group (Fig. 4C–D).

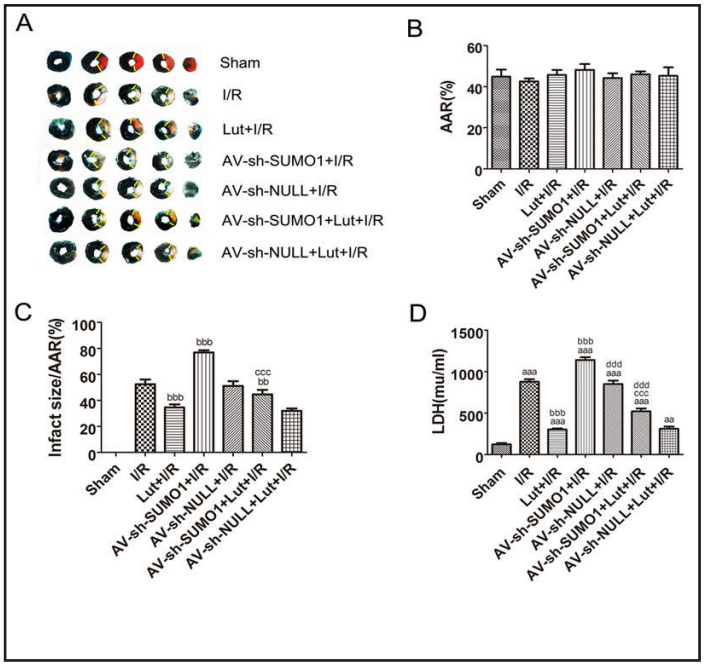

Fig. 4. Detection of myocardial infarction area and serum LDH level. (A) Evans blue staining of each group. Blue-stained is the non-ischemic myocardium. Blue-unstained is the danger area. White is the infarct area. The yellow lines are the ischemic boundary. (B) The results of AAR. Risk area is expressed as the percentage of Evans blue-unstained area to the whole left ventricle area. (C) Infarct size is expressed as the percentage of TTC-unstained area to Evans blue-unstained area. (D) Level of serum LDH in each group. Data are presented as mean ± SEM (n=3). aa $P<0.01$, aaa $P<0.001$ versus Sham; bb $P<0.01$, bbb $P<0.001$ versus I/R; ccc $P<0.001$ versus Lut +I/R; ddd $P<0.001$ versus AV-sh-SUMO1+ I/R.

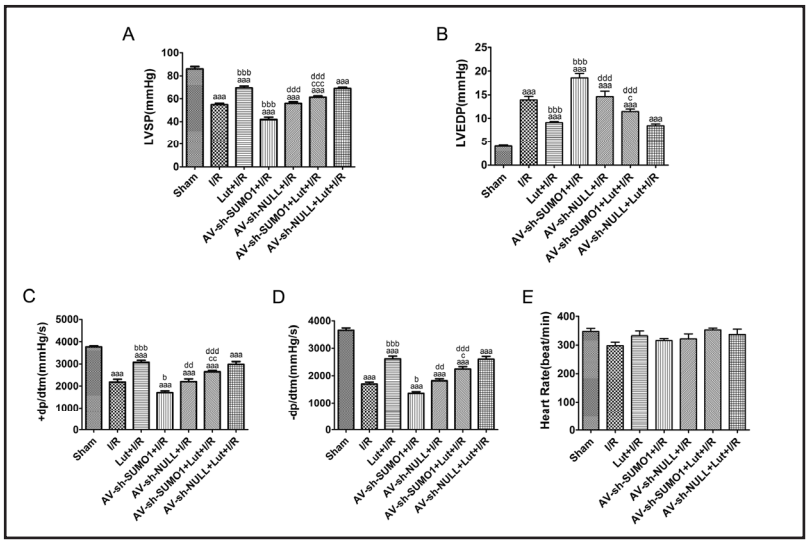

Fig. 5. Changes of cardiac function in each group. (A-E) The results of LVSP, LVEDP, +dp/dtmax, -dp/dtmax and HR respectively. Data are presented as mean ± SEM (n=3). aaa P<0.001 versus Sham; b P<0.05, bbb P<0.001 versus I/R; c P<0.05, cc P<0.01, ccc P<0.001 versus Lut +I/R; dd P<0.01, ddd P<0.001 versus AV-sh-SUMO1+ I/R.

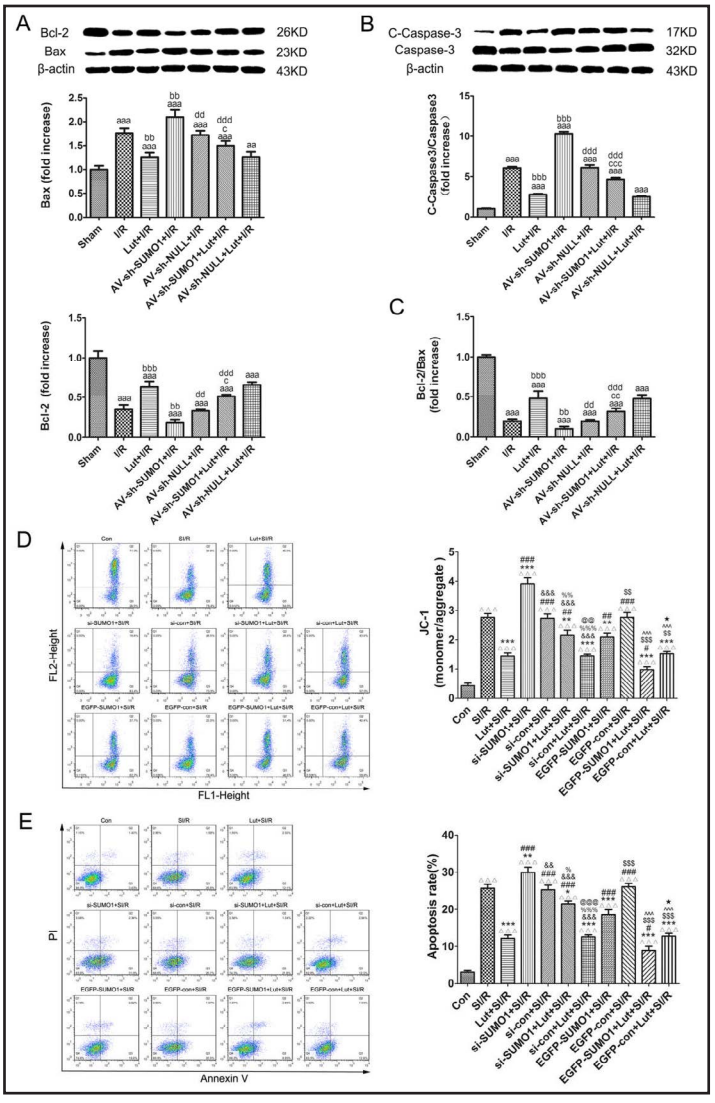

Fig. 6. Lut downregulates cardiomyocyte apoptosis and improves mitochondrial membranepotential (ΔΨm) partly through SUMO1. (A) The protein expressions of Bcl-2 and Bax. (B) The level of C-Caspase-3 protein. (C) The ratio of Bcl-2/Bax in each group. (D) HL-1 cells were pre-treated with siRNA to knockdown SUMO1 and plasmids to over-express SUMO1. Cells stimulated with SI/R following the pretreatment of Lut were stained with Annexin V APC/PI before apoptosis was analyzed by flow cytometry. (E) Analysis of mitochondrial membranepotential (ΔΨm) in each treatment group. Data are presented as mean ± SEM (n=3). aa P<0.01, aaa P<0.001 versus Sham; bb P<0.01, bbb P<0.001 versus I/R; c P<0.05, cc P<0.01. ccc P<0.001 versus Lut+I/R; dd P<0.01, ddd P<0.001 versus AV-sh-SUMO1+I/R; ΔΔΔP<0.001 versus Con; *P<0.05, **P<0.01. ***P<0.001 versus SI/R; #P<0.05, ##P<0.01, ###P<0.001 versus Lut+SI/R; &&P<0.01, &&&P<0.001 versus si-SUMO1+SI/R; %P<0.05, %%P<0.01, %%%P<0.001 versus si-con+SI/R; @@P<0.01, @@@P<0.001 versus si-SUMO1+Lut+SI/R; $$P<0.01, $$$P<0.001 versus EGFP-SUMO1+SI/R; ^^^P<0.001 versus EGFP-con+SI/R; ★P<0.05 versus EGFP-SUMO1+Lut+SI/R.

Regulated SUMO1 expression changed the effect of Lut on cardiac function

I/R injury resulted in a significant decrease in heart function. A significant increase in LVEDP was found, as well as a reduction in LVSP, +dP/dtmax and -dP/dtmax. Pretreatment with Lut significantly improved damaged cardiac function caused by I/R injury. Following knockdown of SUMO1, cardiac function which was improved by Lut was significantly weakened compared with the Lut+I/R group. Compared with the I/R+AV-sh-SUMO1 group, heart function was improved in the AV-sh-SUMO1+Lut+I/R group (Fig. 5).

Up-regulation of SUMO1 by Lut inhibited apoptosis proteins expression in vivo

To investigate apoptosis in each group, the expression of Vcl-2, Bax, Caspase-3 and Cleaved-Caspase-3 (C-Caspase-3) were measured in vivo. I/R promoted apoptosis as it enhanced the expression of pro-apoptotic proteins, Bax and C-Caspase-3, and reduced the anti-apoptotic protein level of Bcl-2 and Caspase-3. Pretreatment with Lut significantly increased anti-apoptotic protein levels and decreased the expression of pro-apoptotic proteins. When compared with the Lut+I/R group, anti-apoptotic proteins in the AV-sh-sumo1+Lut+I/R group were significantly reduced and pro-apoptotic proteins were significantly upregulated. The ratio of Bcl-2 and Bax expression is known to be a determining factor in resistance to apoptosis. We found that this ratio was significantly decreased following myocardial I/R injury, but was enhanced by Lut pretreatment (Fig. 6A–C).

Lut regulates SUMO1 to decline ΔΨm in HL-1 cells

The ratio of JC-1 monomer/aggregate emission is dependent only on the ΔΨm and no other factors. An increase in this ratio reflects low ΔΨm. The exposure of HL-1 cardiomyocytes to SI/R injury resulted in a decline in ΔΨm ($P<0.001$), which partly recovered following Lut pretreatment ($P<0.001$). Si-SUMO1 significantly aggravated the damage to ΔΨm caused by SI/R injury ($P<0.001$), which was partly relieved by Lut pretreatment ($P<0.001$). Moreover, the SI/R-induced decline in ΔΨm was significantly reduced by transfection with EGFP-SUMO1 ($P<0.01$), and the ΔΨm was further increased when cells was treated with Lut and EGFP-SUMO1 together ($P<0.001$) (Fig. 6D).

Influence of Lut on SUMO1 decreased the apoptosis of HL-1 cells

AV/PI dual staining showed an increase in the rate of apoptosis in HL-1 cells induced by SI/R injury ($P<0.001$). Lut pretreatment was found to partly decrease apoptosis ($P<0.001$). Transfection with si-SUMO1 accelerated the apoptosis of HL-1 cells following SI/R injury ($P<0.001$). There were fewer apoptotic cells in the si-SUMO1+Lut+SI/R group than in the si-SUMO1+SI/R group ($P<0.001$). Furthermore, transfection with EGFP-SUMO1 reduced the

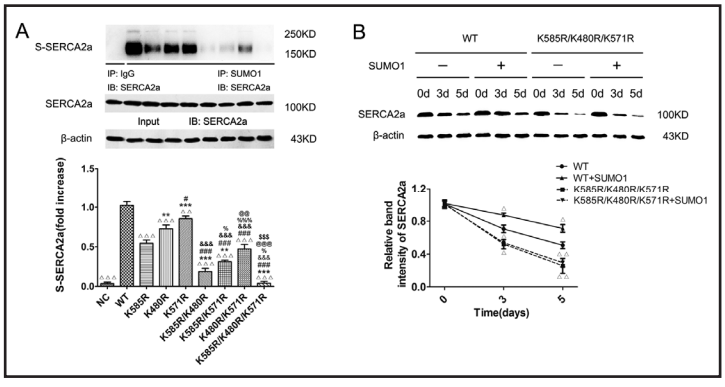

Fig. 7. SUMO1 is conjugated to lysines 585, 480 and 571 of mouse SERCA2a and is required for SERCA2a stability. (A) The evaluation of SERCA2a SUMOylation by Co-IP following transfecting with site mutant plasmids (WT, K585R, K480R, K571R, K585R/K480R, K585R/K571R, and K585R/K480R/571R). (B) Effects of SUMO1 over-expression on the stability of WT and K585R/K480R/571R SERCA2a mutant protein in HL-1 cells. Data are presented as mean ± SEM (n=3). △△P<0.01, △△△P<0.001 versus WT; **P<0.01, ***P<0.001 versus K585R; #P<0.05, ###P<0.001 versus K480R; &&&P<0.001 versus K571R; %P<0.05, %%%P<0.001 versus K585R/K480R; @@@P<0.001 versus K585R/K571R; $$$P<0.001 versus K480RK/571R.

percentage of apoptotic cells induced by SI/R (P<0.001), which was further reduced by pretreatment with Lut following transfection with EGFP-SUMO1 (P<0.001) (Fig. 6E).

Three SUMO conjugation sites in mouse SERCA2a

Three potential SUMOylation sites K585, K480 and K571 of mouse SERCA2a were predicted by the SUMOplot™ Analysis Program (http://www.abgent.com/sumoplot). These SUMO conjugation sites were evaluated by co-immunoprecipitation following transfection with several site mutant plasmids. The single-lysine mutation of K585R, K480R and K571R decreased SERCA2a SUMOylation compared with the WT group, in which the effect of K585R was the most obvious. SERCA2a SUMOylation was further reduced with the transfection of double-lysine mutant plasmids. In addition, the triple-lysine mutant, K585R/K480R/K571R, was completely un-SUMOylated (Fig. 7A).

SUMO1 increases SERCA2a stability

When SUMO1 was co-expressed, the estimated half-life of SERCA2a protein increased compared to the WT. The estimated half-life of the K585R/K480R/K571R mutant was significantly reduced compared with the WT, which could not been reversed by SUMO1 co-expression (Fig. 7B).

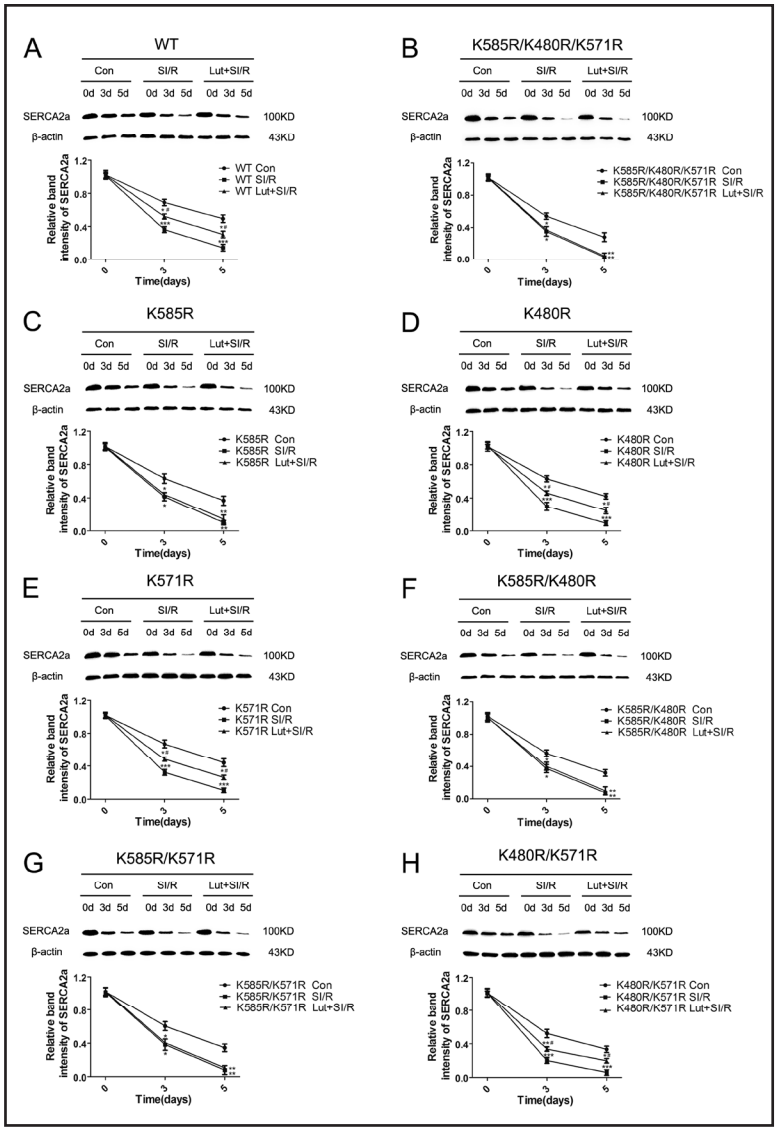

Fig. 8. Lut restores SERCA2a protein stability reduced in SI/R process through SUMOylation at lysine 585. (A) The effects of Lut on SERCA2a stability subject to SI/R injury following transfection with WT. (B) Changes of SERCA2a stability in SI/R process with pretreatment of Lut and transfection with K585R/K480R/571R. (C) K585R abrogates the effect of Lut on SERCA2a stability. (D) Effects of Lut on the stability of K480R SERCA2a mutant protein. (E) The stability of K571R SERCA2a mutant protein in HL-1 cells pretreated with Lut. (F) K585R/K480R ablishes the effect of Lut on SERCA2a stability. (G) Changes of SERCA2a stability in SI/R process with pretreatment of Lut and transfection with K585R/K571R. (D) Effects of Lut on the stability of K480R/K571R SERCA2a mutant protein. Data are presented as mean ± SEM (n=3). *P<0.05, **P<0.01, ***P<0.001 versus the respective Con; #P<0.05 versus the respective SI/R.

Lut stabilizes SERCA2a mainly through K585

Lut increased SERCA2a stability which was reduced by SI/R injury following transfection with the WT (Fig. 8A). However, the K585R/K480R/K571R mutant abolished this effect (Fig. 8B), which indicated that Lut influenced SERCA2a stability by regulating SERCA2a SUMOylation. To study the potential SUMOylation acceptor site(s) Lut acted on, we then determined SERCA2a stability following transfection with other site mutant plasmids. One of these, K585, was critical for SERCA2a SUMOylation. K585R abrogated the effect of Lut on SERCA2a stability similar to K585R/K480R, K585R/K571R and K585R/K480R/K571R (Fig. 8B, C, F, G). However, the effect of Lut was not abolished by K480R, K571R or K480R/K571R (Fig. 8D, E, H).

Discussion

SERCA2a is crucial for maintaining myocardial calcium handling and the systolic/diastolic function of the heart [28]. Heart dysfunction can be caused in the inducible cardiac-specific SERCA2a knockdown mouse [29, 30]. It has been discovered that the expression of SERCA2a was decreased in I/R cardiomyocytes [31]. Our study provided similar evidence that SERCA2a expression declined following I/R injury, but Lut partly restored the level of SERCA2a. Moreover, Lut protected cardiomyocytes against I/R injury, including decreasing apoptosis and improving ΔΨm. It has been reported that with the decline of SERCA2a level, myocardial cells lose their ability to transport calcium from the cytosol into the SR. The abnormal high cytoplasmic calcium level could result in mitochondrial permeability transition pore opening, ΔΨm decrease, and finally cause apoptosis of cardiomyocytes [32]. In addition, Lut can regulate cell apoptosis by enhancing autophagy [33, 34]. These may be the mechanism involved in the protective effect of Lut on I/R cardiomyocytes.

SUMO1 is one of three SUMO isoforms found in the myocardium [35]. SUMO1 usually modifies its substrate as a monomer, whereas SUMO2 and SUMO3 can build up poly-SUMO chains as they are closely related and are usually described as SUMO2/3 [36]. Notably, only SUMO1 binds to two lysine residues of SERCA2a and modulates the level and activity of SERCA2a in cardiomyocytes [37]. In the present study, it was confirmed that Lut restored SUMO1 expression reduced by myocardial I/R injury. Moreover, over-expression of SUMO1 increased SERCA2a mRNA and protein expression, improved ΔΨm and reduced the number of apoptotic cells. As a regulator of SERCA2a, SUMO1 can enhance the function of SERCA2a, but its role was less obvious than the corresponding effect of Lut. Lut may have a protective effect on myocardial cells through other mechanisms. In addition, when SUMO1 was knocked down, the expression and activity of SERCA2a as well as SERCA2a SUMOylation were decreased, and myocardial I/R injury was then aggravated which increased infarction areas, reduced cardiac function, lowered ΔΨm and increased apoptosis, which suggested that SUMO1 has protective effects in myocardial I/R injury. However, the above injury was partly restored by pretreatment with Lut. This recovery may be due to other mechanisms related to the cardioprotective effects of Lut. It is suggested that Lut up-regulated SERCA2a not entirely, but partially via SUMO1.

SERCA2a has been reported to be modified at multiple levels of a signaling cascade consisting of phospholamban, protein phosphatase 1, inhibitor-1, and protein kinase Cα (PKCα) [38]. Our previous study has demonstrated that Lut can improve myocardial contractile function in I/R injury through the ERK/PP1a/PLB/SERCA2a signaling pathway [20], and enhancing SERCA2a activity by p38 MAPK Signaling [39]. In terms of transcription factors, a number of studies have provided compelling evidence that HIF-1α, SP1 and Egr-1 are involved in the regulation of SERCA2a [40–42]. In addition to SUMOylation, SERCA2a may have a cardioprotective effect due to other protein modifications, such as nitration [43], carbonylation [44], and glutathione-based [45]. Moreover, an important mechanism of SUMOylation is competing with other modifications of the target at the acceptor lysine residue. Potential alternative modifications are acetylation, ubiquitylation and methylation

[46]. It has been reported that SUMO modification can compete with other modifications of SERCA2a, especially acetylation [47]. Whether Lut regulates SERCA2a through the above mechanisms requires further investigation.

The SUMOylation pathway plays a prominent role in diverse cellular processes, involving altered localization, activity and stability of the target protein 46. Our previous study has reported that Lut could improve activity and Sumoylation of SERCA2a in cardiomyocytes of heart failure rats [48]. In our present study, we further investigated the effect of Lut on SERCA2a SUMOylation in I/R injury. We found that decreased SERCA2a SUMOylation induced by I/R injury was restored by Lut pretreatment. Furthermore, we proved the above effect of Lut by knocking down SUMO1 in vivo. Additionally, its mechanism was explored in terms of SUMO conjugation sites in SERCA2a. It has been reported that two putative SUMO conjugation sites on human SERCA2a, lysine 585 and 480, are conserved in mouse and human SERCA2a [37]. Nevertheless, we observed lysine 585, 480 and 571 to be SUMO acceptor sites in mouse SERCA2a. Of these, lysine 571 has never been reported, and lysine 585 and 480 were consistent with a previous study. Moreover, we found that Lut improved SERCA2a stability which declined following I/R. However, the K585R mutant abolished this ability of Lut, similar to K585R/K480R/K571R. It is concluded that Lut enhanced SERCA2a stability by regulating SERCA2a SUMOylation mainly at lysine 585. It is known that protein stability is one of the main factors mediating protein expression. The increased protein expression of SERCA2a by Lut may be caused by the above mechanisms. SUMOylation may induce a conformational change [49] in SERCA2a. It is possible that SUMOylation provides an additional interface for ATP binding, leading to increased ATPase activity. It has been reported the control of protein expression through mRNA stability in calcium signalling [50]. Zhang et al. observed that SUMOylation of Nucleolin at K294 mediated cell death by regulating gadd45 mRNA stability [51]. 3-UTR mRNA pull-down assays and Western blot analysis indicated that the AU binding protein AUF1 interacted with the SERCA2a 3-UTR, which affected mRNA stability [52]. Further elucidation needs be explored that whether the effect of Lut on SUMOylation of SERCA2a at K585 has any relationship with mRNA stability. The action site of Lut on lysine 585 might provide a potential therapeutic target for the clinical prevention and treatment of myocardial I/R injury.

The SUMOylation and ubiquitination processes share similarities in terms of the three-dimensional structures of SUMO and ubiquitin proteins. SUMOylation prevents the substrate from depredating by the ubiquitin-proteasome system. In addition, as a reversible and dynamic process, SUMOylation is reversed by a family of Sentrin-specific proteases (SENPs). SENP1 can protect against myocardial I/R injury [53]. Further experiments are required to determine whether Lut increases SERCA2a stability by reducing its ubiquitination, and whether SENP1 is involved in the mechanism by which Lut attenuates myocardial I/R injury.

There are several vital findings in the present study. First, Lut upregulates SERCA2a partly through SUMOylation to attenuate myocardial I/R injury. Second, Lut increases the protein stability of SERCA2a by improving its SUMOylation, which contributes to the upregulation of SERCA2a. Third, the putative SUMO conjugation sites in mouse SERCA2a include lysine 585, 480 and 571 Of these, lysine 585 is the main SUMO acceptor site by which Lut affects SERCA2a stability. These findings may represent a novel strategy by which Lut protects the heart from I/R injury and this strategy requires further investigation.

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Disclosure Statement

The authors declare that they have no competing interests.

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